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Systematic analysis of carbon nanotubes toxicity in cellular systems

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Systematic analysis of carbon nanotubes toxicity in cellular systems

By

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Thesis submitted to the Benjamin M. Statler College of Engineering and Mineral Resources at
West Virginia University

in partial fulfillment of the requirements for the degree of

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in

Chemical Engineering

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Abstract

As the applied use for carbon nanotubes (CNTs) increases across engineering and biomedical sectors, a clear understanding of the deleterious effects surrounding CNT-induced toxicity, namely genotoxicity, is required. Currently, the exact genotoxic mechanisms that surround CNT-induced mitotic disruption that result in aneuploidy are not completely understood. Thus, there is a need for clear mechanical models to be formulated. Herein a comprehensive overview of the nanotube toxicity is provided insisting on the aspects related to CNTs genotoxicity.

In Chapter 1, I am providing a review of the current research in the CNT-s induced toxicity. I start by defining these members of the fullerenes family and subsequently talk about their implementation in a variety of applications from interconnects to composite polymers, from electrodes to high temperature fillers, from bioimaging to biosensors, and finally in drug delivery. Next, I introduce aspects related to the CNT toxicity *in vitro* and *in vivo*, both in relation to the cytotoxicity and genotoxicity specifically focusing on what is currently known about CNT-induced genotoxicity and suggesting more research in the area of critical mitotic mediators i.e. the cytoskeletal filament, microtubules and their associate mitotic molecular motors, while also proposing an application for CNT use in targeted drug delivery, respective of molecular motor targets. Lastly, my Chapter focuses on the current research in CNTs-induced genotoxicity and proposes a more exact model of the aneuploidy that could take place upon cellular exposure. Such findings could lead to designing novel CNT-based platforms to improve chemotherapeutic efficiencies for targeted delivery and cancer therapeutics. However, this Chapter also points out that the goal for CNT use in cancer therapy will require convergence to minimize host cytotoxicity, while maximizing cancer cell genotoxicity.

In Chapter 2, I describe functionalized MWCNT-induced mitotic disturbances following *in vitro* exposure of BEAS-2B, human bronchial epithelial cells. I present distinctly different characterizations between the degree of MWCNT functionalization and how that affects the cellular response. Following cell treatment changes in the cell cycle have been observed, resulting in cell cycle arrest, that correlate with changes in mRNA expression of molecular

motors, dynein and Eg5, resulting in a reduction of total protein expression in a time dependent manner. This study is the first to show MWCNT-induced disruptions of critical mitotic mediators, i.e. molecular motor proteins dynein and Eg5, and one of several studies that confirm clear disruptions in the cell cycle following exposure to functionalized MWCNTs, thereby providing a viable mechanical model for the CNT-induced genotoxicity.

Dedication

In loving memory

Douglas Bruce Rogers

(December 13, 1984 – January 30, 2011)

“Forever in our Hearts”

To my children, Lorelei and Jack

“There are great survivors and helpless victims on the curve of human ability. Most of us are neither. Most of us fall somewhere in between and may perform poorly at first, then find the inner resources to return to correct action and clear thought. If the object of the game is survival, that will do.”

Laurence Gonzales “*Deep Survival*”

I will stop at nothing to ensure there is hopefulness and happiness in the very bright futures that await you; I encourage you to do the same.

I love you more than infinite space, you have given meaning to my life.

Love Always and Forever

Your Mommy, Gabrielle

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I also want to acknowledge the helpful insight of my peers, and thank, Reem Eldawud, Chenbo Dong and Alan Campbell; the future's bright scientific minds. Additionally, I want to thank the Department of Chemical Engineering faculty and staff for giving me an opportunity to improve my quality of life with further education. I have never worked so hard or been so mentally challenged in my life; it is a completely gratifying experience to know that I have accomplished this task and that I had fun in the process. I have nothing but the highest respect for this department; it does what it does very well.

Lastly, I have to acknowledge and thank my support system; my husband Eric, my mom Theresa, my dad Robert, my brother Douglas, my sister Paola, and my children Lorelei and Jack, as well as all other family and friends who supported me throughout this process; all this would not have been possible if not for your love. This program taught me a lot about inner challenge, but my family taught me more about inner belief. The latter was my hardest lesson, but ultimately the most important one.

God has blessed me beyond belief, and I am eternally grateful.

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Chapter 1

The exploitation of subtle genotoxic associations between carbon nanotube, microtubules, and molecular motors for possible use in cancer therapeutics

Abstract

Carbon nanotubes (CNTs) have been proposed as the next generation vehicles to be used in cancer therapy. Based on their thermal, optical, electrical and mechanical properties that allows for targeted delivery ¹, their large surface area that allows for efficient drug loading ^{2,3}, as well as their ability to localize and be visualized at the tumor site ^{4,5}, CNTs can induce effective killing of tumor cells ⁶ by cell lysis ¹. Even though the potential for novel CNT-chemotherapeutic platforms may exist ⁷, research needs to proceed with caution and account for their cytotoxic and genotoxic mechanisms. Herein we review the recent advances in understanding the toxicological aspects related to carbon nanotube cellular exposure and highlight the roles of structural cellular elements in cellular genotoxicity. Further, we suggest novel means to investigate and exploit the potential of carbon nanotubes to serve as the next generation of tailored chemotherapeutics that actively inhibit cancer cell development.

Introduction

Current cancer therapeutics lack the specificity to target only cancer cells and instead cause toxicity throughout the whole body ^{8,9} thus patients succumb to death ¹⁰. The early detection of host toxicity presents a multifaceted challenge for healthcare professionals on a runaway time axis ¹¹. Further, current in-use chemotherapeutics are being challenged by drug-resistance ¹², as is the case with taxol, a chemotherapeutic that targets the cytoskeletal filament microtubule (MT) ¹³ with roles in cellular division. Additionally, short resonance times ¹⁴ and low dispersity ^{15,16} play a role in the greater inefficiencies of traditional chemotherapeutics. A more effective strategy capable of targeting tumor cells without affecting healthy cells is needed in order to tailor individual treatments.

CNTs are members of the fullerene families and allotropes of carbon¹⁷⁻¹⁹. CNTs are known for their high aspect ratios, with typical diameters ranging from 1-100 nm and lengths up to several centimeters¹⁸. CNTs are categorized as single-walled or multi-walled carbon nanotubes (SW- or MWCNTs), where SWCNTs refer to a single rolled graphene sheet with diameters ranging from 0.4 to 3 nm, while MWCNTs refer to multiple rolled graphene sheets stacked inside one another and have diameters ranging from 2 to 100 nm¹⁷⁻²³. CNTs are stiff and resilient structures with an average Young's modulus of >1TPa²⁴. This resilience allows the nanotubes to be exposed to extreme “bending” torsion or “sliding” shear forces and return to their original shape without reaching material failure^{22,25-29}. The unique combination of CNTs properties and their intrinsic mechanical²² and electrical properties^{20,21,23}, make the nanotubes ideal candidates for a wide variety of applications from interconnects in micro-electronic devices^{30,31}, to oxide semi-conductor field effect transistors (MOS-FET)³²⁻³⁴, from electrodes for lithium-ion batteries³⁵⁻³⁸, to load-bearing reinforcements in composites³⁹⁻⁴³, and from high temperature fillers⁴⁴⁻⁴⁶, to microcatheter polymer composites able to reduce thrombogenesis and blood coagulation⁴⁷. CNTs recent functionalization with biological molecules such as DNA^{48,49}, RNA⁴⁸, enzymes^{50,51} and proteins⁵² have led to nanotubes being explored as novel, and innovative tools with applications in bioimaging^{53,54}, on biosensor platforms⁵⁵, and for targeted delivery of drugs^{56,57}. A summary of the applications of CNTs in synthetic environment is shown in Figure 1.

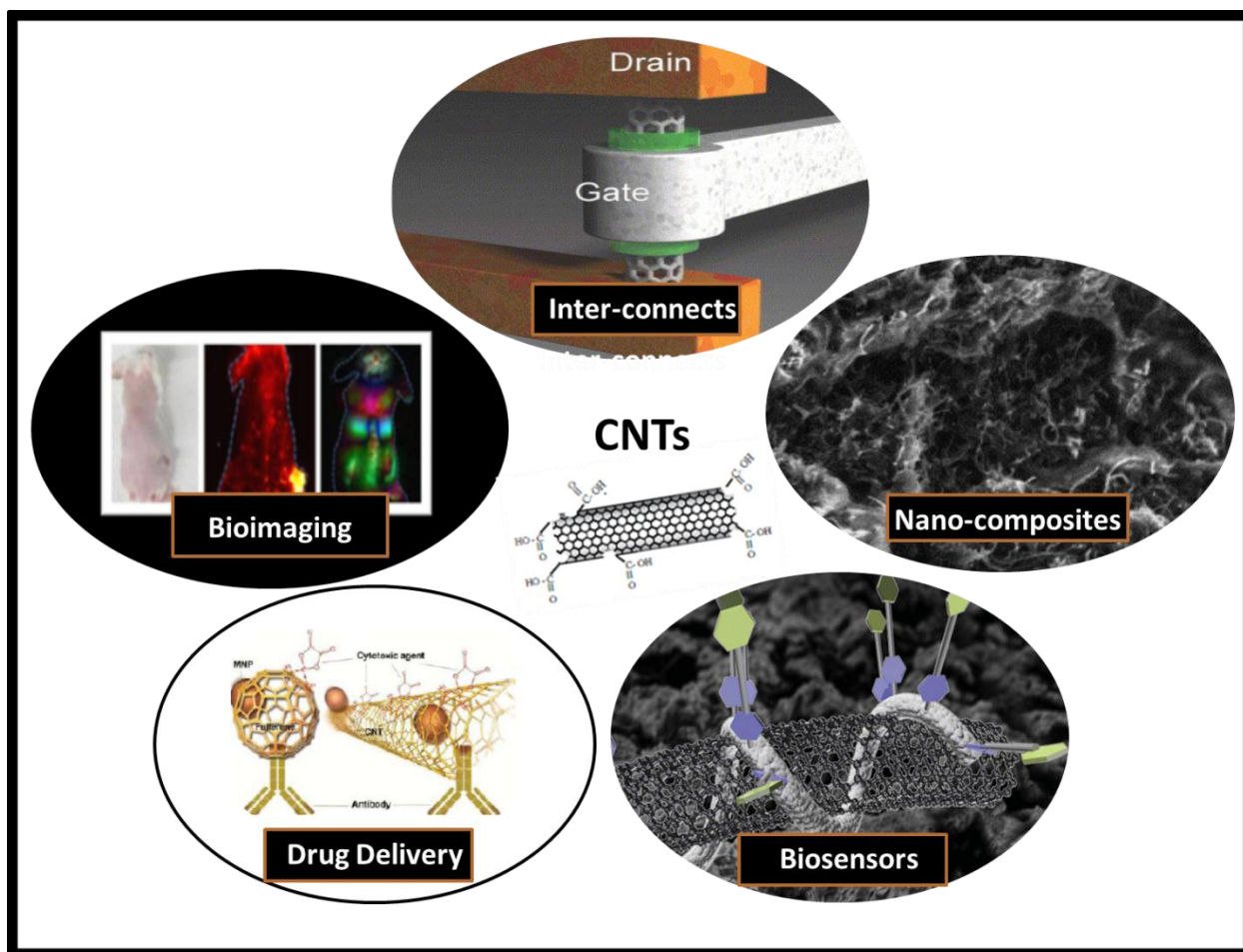


Figure 1: The schematic diagram shows the emerging engineering applications of carbon nanotubes (CNTs): **Bioimaging**, figure adapted from Robinson et al.,⁵⁴ doi: 10.1021/ja303737a, **Drug Delivery**, figure adapted from Omid et al.,⁵⁸ doi: 10.5681/bi.2011.028, **Biosensors**, figure adapted from Shi et al.,⁵⁹ doi: 10.1039/C1AN15179G, **Microelectronic Inter-connects**, figure adapted from Graham et al.,³¹ doi: 10.1016/j.diamond.2003.10.080, and **Nanocomposites**, figure adapted from Fan et al.,⁴³ doi: 10.1016/j.mseb.2006.02.045.

As carbon nanotubes (CNTs) make a fast break into the vast world of nanotechnology, so follows our interest to harness their exceptional properties. The question remains, can we use CNTs for cancer therapy? In this article, we first review the current status of available methodologies for applications of CNTs in the biomedical engineering and biotechnology; subsequently we discuss the toxicological aspects related to CNT exposure in biological systems and define implications of CNT-induced genotoxicity, and lastly propose viable means to exploit CNTs properties for designing CNT-based platforms for tailored cancer therapeutics.

Carbon nanotubes in bio-related disciplines

In bioimaging, Khandare et al., have shown that MWCNTs conjugated via polyethylene glycol (PEG) spacer to fluorescein isothiocyanate (FITC) have a faster cellular uptake and reach the perinuclear area of the breast cancer cell line, MCF7, cells faster than FITC alone ⁶⁰. Cherukuri et al., have explored SWCNT intrinsic fluorescence to demonstrate uptake in macrophages ⁶¹. For biosensors applications, Pan et al., have shown that immobilization of CNTs with glucose oxidase (GOD), and poly(o-aminophenol) (POAP) on gold(Au) electrodes, increased sensitivity of an amperometric glucose biosensor, resulting in lower detection limits, and thus larger responses when compared to GOD/POAP/Au electrodes alone ⁶². Further, Kurkina et al., reported on the use of CNTs in an electrochemical biosensing platform for the detection of low target DNA sequence concentrations for point of care applications. In this particular study, SWCNT-based biosensors showed, on order of magnitude lower detection capability when compared to currently used silicon nanowires ^{63,64} for target synthetic DNA sequences that comprised only 2% of the total DNA pool ⁶⁵. Lastly for drug delivery, Wu et al., showed MWCNTs functionalization with amphotericin B (AmB), an antibiotic that treats fungal infections and is otherwise toxic to mammalian cells if used free in solution. In their study, the CNT-AmB conjugates showed effective killing capability of *C. parapsilosis*, *C. albicans*, and *C. neoformans* fungi colonies, with no simultaneous toxicity towards Jurkat T-lymphocytes cells ³. Complementary, Liu et al., showed that SWCNT-taxol conjugates, significantly decreased tumor weight and increased apoptosis in mouse T41 breast cancer *in vivo* models when used for cancer therapy. The authors also observed a 10-fold higher uptake of these conjugates in the tumor cells ². However, despite these examples that show CNTs implementation in biomedical engineering ^{66,67} and biotechnology ^{53,55-57}, there are several factors that hinder CNTs full potential for use as therapeutic vector agents for drug and gene transfection into cellular systems ⁶⁸. Further, while carbon nanotubes have been vied as the “wonder solution” for cancer therapeutics ⁷, a clear understanding of how they effectively kill, mechanistically, still remains ill defined ⁶⁸. These limitations are associated with the lack of consistency in toxicological and pharmacological studies that assess CNTs relatable exposure hazards and how such exposures do or do not lead to cellular harm ⁶⁹.

Cytotoxicity associated with CNT exposure

Exposure hazard and toxicity related to CNTs are likely to occur in production and manufacturing settings where nanomaterials can aerosolize and aggregate into a concentrated area ⁷⁰⁻⁷³, and thus be easily inhaled ⁷⁴⁻⁷⁸. Current exposure limits for CNTs fall under the category of “particles not otherwise regulated” at a 5mg/m³ exposure concentration ⁷⁹. The primary models used for detecting work-relatable exposures are from inhalation studies using rats and mice. The recommended exposure limit (REL) of 7µg/m³, was calculated assuming 8 h work shifts, 40 h per week work schedules for 50 weeks per year for a duration of 45 years ⁸⁰. Further *in vivo* rat and mouse acute and sub-chronic studies ^{74-78,81-84} provided evidence for the determination of the benchmark dosage (BMD) for human exposures by accounting for differences in alveolar lung surface area ⁸⁰. Research has shown that following inhalation of particles of low solubility two distinct pathways are responsible for induced toxic changes. The primary path depends highly on the intrinsic activity of the particles themselves, while the secondary path is relative to the inflammatory events that are elicited following exposure to the tissue ⁸⁵.

Across *in vivo* and *in vitro* studies, CNT type, functionalization, dispersion,^{56,86} and dosage all seem to relate to toxicity, and in cases, have led to conflicting reports. A summary of cyto- and genotoxicity of both SW- and MWCNTs is shown in Figure 2. For instance, some studies have suggested that functionalized CNTs i.e. low metal, high carbon compositions versus non-functionalized or pristine CNTs i.e. high in metal impurities at low dosages, are free of cytotoxic insult to cells ⁸⁷⁻⁹⁰. Other studies have however shown that following exposure to either SW- or MWCNTs, pulmonary toxicity ⁷⁴⁻⁷⁸ and the potential for carcinogenesis is induced ^{91,92}. *In vivo* studies have shown that CNT exposure leads to granuloma formation ⁷⁶⁻⁷⁸, pulmonary fibrosis ⁷⁶⁻⁷⁸ involving bronchiolar and alveolar epithelial cells, formation of reactive oxygen species (ROS) ^{83,93}, up-regulation of the innate immune system and observation of macrophages with multiple or no nuclei ⁸¹. *In vitro* studies have shown disruption of the mitotic spindle ⁹⁴, ROS generation, ^{90,95,96} up-regulation of pro-inflammatory pathways ^{95,97-99} as well as micronuclei formation ^{93,100,101} and fragmentation of DNA ^{96,101-103}. Ding et al., have shown that exposure to high concentrations of MWCNTs led to quick innate immune responses through type I interferon (IFN) signaling. Such a response up-regulated several pro-inflammatory cytokines,

including but not limited to, tumor necrosis factor-alpha (TNF- α), BCL-like-protein 2 (BCL2L2) and induced myeloid leukemia cell differentiation protein-1 (MCL1), a key regulatory protein of apoptosis⁹⁷. Additionally, generation of ROS following treatment with SWCNTs was shown to lead to impaired DNA repair function and subsequently to apoptosis through poly-ADP-ribose polymerase (PARP) cleavage^{96,97}. Further, it appears that SWCNT treatment stimulates activation of important transcription factors i.e. activator protein-1(AP-1) and nuclear factor-kappa light chain enhancer of activated B cells (NF- κ B) which are mediated by upstream kinase regulation through stimulation of extracellular signal regulated kinases (ERKs), Jun-N-terminal kinases (JNKs) and protein p38⁹⁶. These analyses suggested that the induction of p38/ERK pathway and the type 1 IFN signaling response are responsible for down-stream transcriptional changes following exposure^{96,97}.

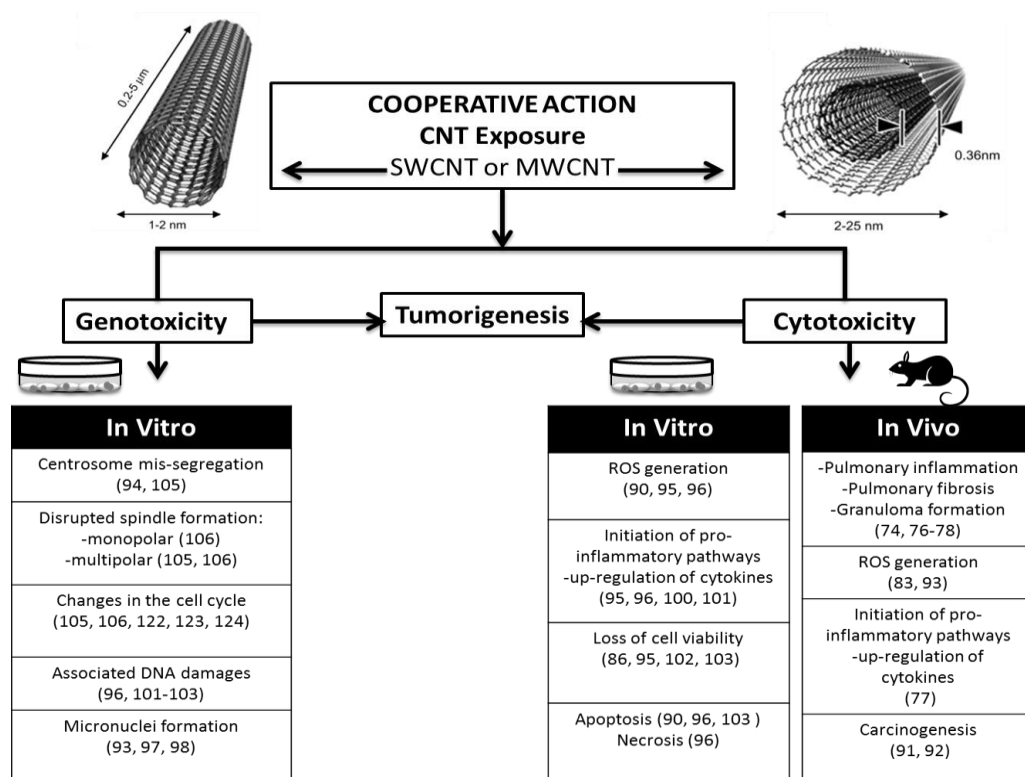


Figure 2: *In vivo* and *In vitro* exposure to carbon nanotubes (CNTs) lead to cyto- and genotoxicity, a summary of the toxicological mechanisms induced by single-walled carbon nanotubes (SW- and MWCNTs). CNT (SW- and MWCNTs) figures adapted from Raymond Reilly¹⁰⁴ doi: 10.2967/jnumed.107.04.1723.

A closer look at the mechanisms of CNTs-induced cellular genotoxicity

Studies following treatment with either SW- or MWCNTs have also reported observable changes in normal cell cycle progression. For instance, following exposure to either SW-¹⁰⁵ or MWCNTs¹⁰⁶, cells were observed leaving G1 phase, likely following mitotic “slippage”, only to subsequently sequester in S/G2 phases in response to treatment¹⁰⁷. Mitotic slippage occurs when cells escape mitotic arrest and subsequently divide, without even distribution of genetic copies to progeny cells. Further, the cells enter the G1 phase and no further cellular division occurs¹⁰⁷. Studies involving SWCNTs showed nanotube integration with the cellular elements and disruption of the cellular centrosome¹⁰⁵ (Figure 3A), with similar effects of aberrant mitotic spindles observed for MWCNTs (Figure 3B)¹⁰⁶. Other reports indicate global DNA damage¹⁰⁸, or inhibition of the mitotic spindle assembly and movement^{109,110}.

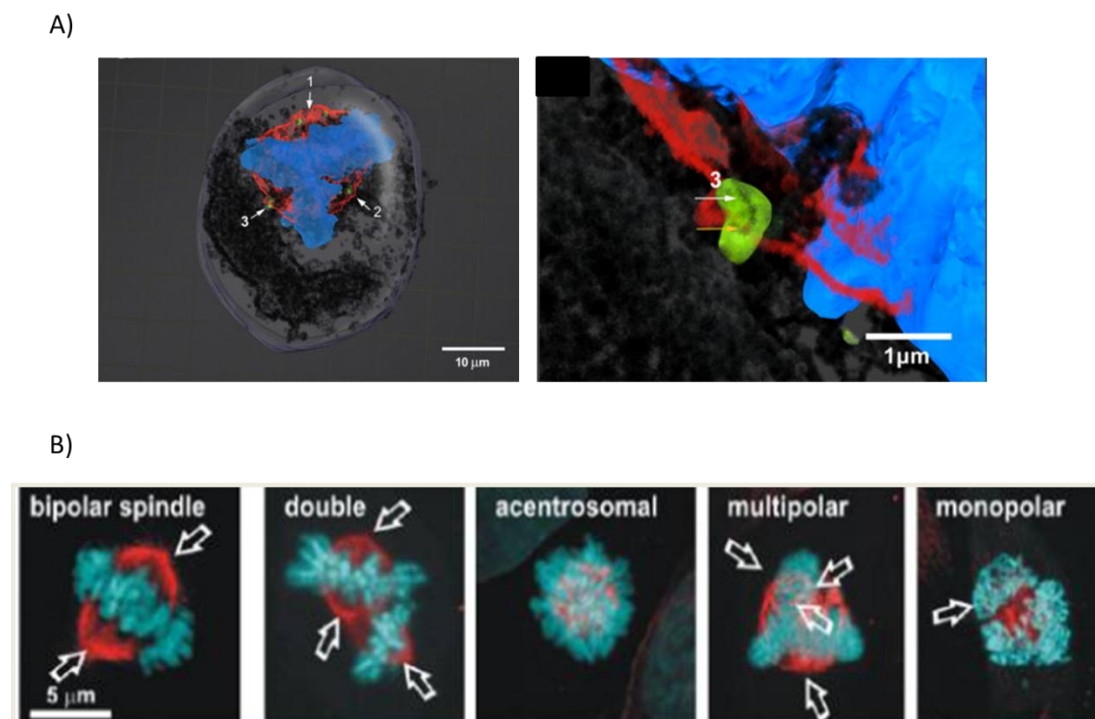


Figure 3: A) A 3D reconstruction of the multipolar mitotic spindle with three poles. The DNA is blue, the tubulin is red, the centrosomes are green and the nanotubes are black. The three spindle poles are indicated by white arrows. Serial optical sections of 0.1 μm in depth were used to construct a 3D image of the tripolar mitosis. The reconstructed image shows nanotubes inside the cell in association with each centrosome fragment at the 3 spindle poles. Nanotubes are also

integrated with the microtubules and the DNA. In the centrosomes and the portion of the mitotic spindle labeled as region 3 in are increased in size to show details of the nanotube association with the centrosome and the tubulin. The nanotubes can be seen within the centrosome structure as indicated by the white arrow. The nanotubes associated with the microtubule can also be seen in more detail as indicated by the yellow arrow. In this cell, the three spindle poles, the three unequal DNA bundles, and the disruption of microtubule attachments to two centrosomes suggest major perturbations in cell division, , figure copyrighted from Sargent et al., ¹⁰⁵ doi: 10.1016/j.mrgentox.2011.11.017 . B) Confocal microscopy projection images of aberrant mitotic spindles in cells treated with MWCNTs. Aberrations in the organization of the spindle microtubules (red channel) and chromosomal distribution (blue channel) are observed, figure copyrighted from Rodriguez-Fernandez et al., ¹⁰⁶ doi: 10.1021/nn302222m.

Mitotic spindles formation ¹¹¹ is driven by the integration of the structural framework of microtubule (MT) cytoskeletal filaments ^{112,113}. MTs are formed by polymerization of α and β subunits of tubulin¹¹⁴. The α and β tubulin dimers orient in a head to tail fashion and lead to protofilaments formation; 13 protofilaments associate laterally to form the MT structure ¹¹⁵. Both subunits bind guanine triphosphate (GTP), however only the GTP bound to the β -subunit is hydrolyzed during polymerization. This allows for fast addition of other β -subunits and thus a “fast growing” or “plus end” of the microtubule. The GTP bound to the α -exposed end is not hydrolyzed, thus making the α -exposed end the “slow growing” or “minus end” of the MT structure ¹¹⁵. MTs have an average diameter of 25 nm, and make up the “tracks” used by the molecular motors for organelles movement throughout the cell during interphase ¹¹⁶. Specifically, the MTs begin to nucleate, grow, and organize prior to centrosome duplication and separation ^{111,117,118}. MT nucleation starts at the microtubule organization center (MTOC) with γ tubulin ring formation encapsulating the minus end of the MTs, followed by the α and β -subunit polymerization ¹¹¹, and finally orientation of the MT plus ends towards the cell periphery. ^{111,119} Later, beginning in prophase, MTs make up the framework for the mitotic spindle formation ¹¹⁶.

MWCNT exposure to cells *in vitro* have been described as both clastogenic, leading to disruption or breakage of chromosomes observed by formation of micronuclei, and aneugenic, whereby exposure affects cellular division and mitotic spindle formation ⁹³. Muller et al. 2008, reported that MWCNT exposure causes both clastogenic and aneugenic effects that lead to whole

or partial loss of chromosomes ⁹³ similar to known carcinogen crocidolite asbestos ^{96,120,121}. Further, the display of a mitotic checkpoint response following treatment with either SW ¹²²-or MWCNTs ¹²³ and blockage in normal progression toward cell proliferation suggests that CNTs treatment may also result in impaired phosphorylation cascades, and/ or deviations in transcriptional regulation of important cell cycle regulators. As seen by Zhang et al., treatment with carboxylated MWCNTs mediates over-expression of cyclin-dependant kinase inhibitor 1 (p21), a known inhibitor of CDK1 & 2, and is independent of tumor protein 53 (p53) protein expression via impaired bone morphogenic protein (BMP) signaling, suggesting that p21 itself mediates cell cycle arrest in response to treatment ^{123,124}. Further, Nam et al., have shown a significant reduction in CDK2 and CDK6 cell cycle proteins in normal rat kidney epithelial cells treated with sodium dodesoyl sulfate (SDS) functionalized SWCNTs. In this study, reduced phosphorylated-retinoblastoma (pRB) and lowered cell viability, as well as increased p53 and p21 expression and growth arrest at the G0/G1 phase border was observed ¹²². However, the exact mechanisms that lead to defects in the mitotic spindle and results in aneuploidy following CNT exposure have yet to be fully explained.

The role of microtubules and molecular motors in CNTs-induced genotoxicity

CNTs have been observed in proximity to cellular and mitotic tubulin, in the mid-body region separating two daughter cells, and in association with MTs ^{94,105,106}. Further, *in vitro* polymerization of tubulin onto CNTs scaffolds led to biohybrid formation ⁵². Such biohybrids consisting of bundles of CNTs encapsulated by augmented MTs with impaired microtubule function ¹²⁵. Taking into account the association of the tubulin with CNTs, as well as previous observations of the inhibition in mitotic spindle assembly movement ^{109,110} and abnormal spindle formation as result of CNT treatment ¹⁰⁵, a closer look on the role that CNTs play in mitotic spindle assembly and disassembly is required. In particular, studies are required to evaluate how nanomaterial-MT-molecular motor associations can affect mitotic assembly and cell division ^{94,105,106,126}.

In eukaryotic cells, dynein, Eg5, Kin14 and Kin4/Kin10 are mitotic molecular motors required to achieve meaningful cellular division ¹²⁷. These molecular motors move along the MT in a directional way following ATP hydrolysis at their respective motor domains and participate in the cellular division process ^{128,129} generating directional force to segregate two daughter cells

^{129,130}. A representation of the cellular mitotic spindle and the molecular motors involved in spindle formation is shown in Figure 4A. For instance, during prophase dynein walks towards the minus end of the MTs and in association with dynactin ^{119,131-135} is responsible for providing the pulling force necessary to segregate replicated centrosomes to either polar end of the cell ^{136,137}. Further, through force oscillations ¹²⁶ with Eg5 the two motors provide mitotic spindle stability throughout mitosis ^{117,118,138-140}. Eg5 is a member of the Bim-C family, a homotetrameric plus end directed molecular motor known to be essential for normal division of cells ¹⁴¹⁻¹⁴⁵. Under ATP hydrolysis Eg5 slides the anti-parallel, inter-polar MTs; this movement is responsible for providing a pushing force to keep the two centrosomes at their respective polar ends ^{141,143-147}. Additional roles of Eg5 have been studied in relation to astral MTs where Eg5 movement towards minus end was observed in early mitosis, which coincidentally, required binding to dynein to aid in centrosome segregation following nuclear envelop breakdown ¹³¹. Kinesin-14 is known as C-terminal or Ncd kinesin, also binding inter-polar MTs, like Eg5, with Kin-14's movement proceeding toward the minus end of the MT ^{148,149}. The ability to slide the force generating inter-polar MTs toward the minus end with Kin-14 and the plus end with Kin-5 ensures proper mitotic spindle balance for equal chromosome segregation during cell division ¹¹¹. Kinesin 4 and 10 are known as chromo-kinesins and are found in all eukaryotic cells. These kinesins associate with the arm of the chromosome and their movement under ATP hydrolysis is directed toward the plus end of the kinetochore MTs, which helping with chromosomal positioning along the metaphase plate ¹⁴⁹.

Inhibition of such molecular motors has shown to have similar effects to exposure to CNTs. In particular, following Eg5 inhibition, monopolar spindle formation ¹⁵⁰ has been observed. Further, studies have shown that when Eg5 is inhibited either by Eg5 siRNA down-regulation ^{151,152}, or by administration of small molecule inhibitors ^{91,107}, disruptions in mitotic spindle formation occur. Inhibition also results in an “arrest-like” status via abrogation of checkpoints leading to mitotic catastrophe, similarly to cellular exposure to nanomaterials. Thus, CNTs interaction with MTs and molecular motors involved in cellular division may lead to mechanical force imbalances within the spindle assembly and alteration of key regulatory pathways that affect protein transcription and expression ⁹⁷⁻⁹⁹, which ultimately lead to aneuploidy ^{93,94} a precursor to early tumorigenesis.

Based on these findings, we can begin to piece together a more exact model for CNT-induced genotoxicity (Figure 4B). A timeline will be required to show how early CNT-induced phosphorylation impairment leads to down regulation of mitotic motor expressions, which then disrupts the formation of the mitotic spindle, while at the same time pushing the cell toward cell cycle arrest. In addition, an *in vivo* model will be required to provide evidence about how CNT treatment can affect the normal association and movement of mitotic motors along MTs. Thus, in addition to the traditional approaches of investigating genotoxicity and the events that lead to CNT-induced aneuploidy ¹⁵³, research should also focus on observing and quantifying CNT-MT and CNT-molecular motor protein associations in real time. For a better description of disruptions in mitotic spindle formation, possibly through altered force oscillations via load-accelerated protein-protein dissociation ^{129,154} we must first observe real time changes, respective of both MTs and molecular motor proteins, as a result of exposure with CNTs.

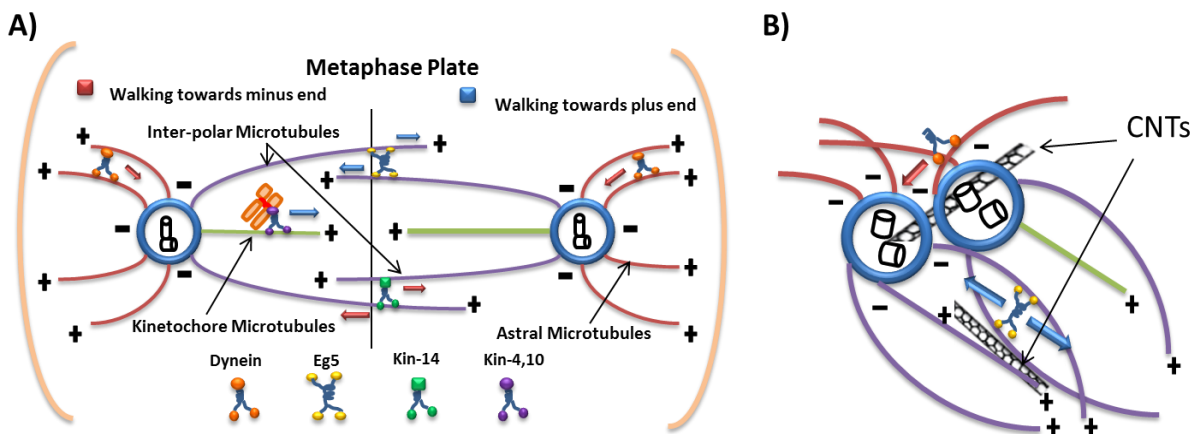


Figure 4: A) A diagram of the eukaryotic cell mitotic spindle: There are three distinct types of microtubules (MTs): Kinetochore MTs bind to the minus end of the centrosome and then attach to the kinetochore of the sister chromatids at the MT plus end, Inter-polar MTs bind to the minus end to the centrosome, and then overlap in the mid-zone region of the mitotic spindle. Inter-polar MTs do not have a chromosomal attachment point. Astral MTs bind to the minus end of the centrosome, and then plus ends grow outward toward the cell cortex. Additionally, the eukaryotic mitotic molecular motors, Dynein, Eg5, Kinesin-14, and Kinesin-4 and 10 move in association with MTs via ATP hydrolysis, and provide directional movement required for proper

spindle formation. Under normal cell cycle conditions MTs and mitotic molecular motors work in concert to achieve bipolar spindle assembly. B) Following CNT exposure, CNTs can interact with the centrosomes, the microtubules and mitotic molecular motors to inhibit proper spindle formation. Lack of association of the mitotic molecular motors with the CNTs-microtubule biohybrids could lead to mis-positioning of the centrosomes and genotoxicity.

Making the best of the worst: CNT use in cancer therapy

Can we use CNTs for the better? Can we envision using their interactions with the molecular motors and microtubules when designing novel CNT-based platforms to improve chemotherapeutic efficiencies by way of higher dispersities, and more targeted delivery? ⁷ Deciding on whether the reality for CNT use in cancer therapy is hopeless or not, is a matter of perspective. If we consider association of CNTs with MTs and molecular motors, both *in vitro* and *in vivo* that leads to cyto- and genotoxicity, we may say “no”. However, if we consider association of CNTs with MTs and molecular motors that escape cytotoxic pathways while at the same time effectively targeting genotoxic pathways, in addition to exploiting CNTs properties for improved dispersity, drug-loading, and targeted delivery potential, then we may say “yes”. Thus, while ensuring that we minimize the costs/risks involving CNT implementation into chemotherapeutics we can at the same time suggest a compromise to achieve greater benefits i.e. better therapeutic outcomes. By designing a more “global” approach in which the user will be 1) inhibiting cancer cell cycle regulatory cascades, 2) inducing down regulation of mitotic motor protein expression, and 3) impairing the ATP hydrolyzed directional movement of motors along MTs, one can program cancer cell death or rather cell cycle arrest for cancer cells only. The key here is to find an effective dosage concentration range relative to the type of CNT, an optimal functionalization for the CNT surface, and achieve full dispersion of CNTs in the dosing media. The goal for CNT use in cancer therapy will require convergence to minimize host cytotoxicity, while maximizing cancer cell genotoxicity.

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Chapter 2

Changes in the Eg5 and Dynein Expression Profiles following Exposure of Epithelial Cells to Multiwalled Carbon Nanotube

Abstract:

Carbon nanotubes (CNTs) have been studied for their potential use in cellular delivery of molecules, drugs and RNA. However, contradictory reports on CNTs toxicity make such applications questionable. Recent studies have shown that upon exposure, CNTs associate with cellular elements, namely the cytoskeletal filament microtubules and DNA, and interfere with the cell progression through normal division most notably by disruption of the centrosome. We hypothesized that CNTs could induce changes in the expression level of the CNTs-treated BEAS-2B human airway epithelial cells, which we used as a model system. Such changes are correlated with changes in the cell cycle progression and draw a parallel relationship with the nanomaterial properties, both physical and chemical. It is anticipated that DNA damage is augmented by interactions of CNTs with the molecular motors and microtubules leading to cell cycle arrest in the G1/S phase. We found that occupational exposure doses induced down regulation in the expression level of key molecular motors involved in cell division, thus representing a newly recognized mechanism by which CNTs may modulate epithelial cell responses germane to genotoxicity and tumorigenesis. We propose that the mechanism involves alterations in the forces responsible for the mitotic spindle formation thus implicating a mechanical imbalance. Such interference could lead to aneuploidy and tumorigenesis.

Keywords: mitotic spindle, Eg5, dynein, genotoxicity, functionalized, MWCNT, cell cycle, *in vitro*

Introduction:

Normal progression through the cell cycle is critical for the maintenance of cellular division, protein expression and cell viability¹⁻³. Cell cycle is regulated through a series of checkpoint responses; efforts to modulate the cell cycle are required to ensure the quality and

rate of cell division. Generally, the loss of cell cycle checkpoint responses has been reported to lead to propagation of unequal chromosomes, irreversible genetic disturbances, and initiation of tumorigenesis^{1,4}. Further, lack of reliability in cellular DNA replication or in the maintenance of the cell cycle and responsible proteins involved with cell cycle progression, can result in deleterious mutations leading to genotoxic stress⁵, cell death^{6,7}, or initiation of cancer^{1,4}.

Changes in the cell cycle have been reported upon cellular exposure to nanomaterials⁸⁻¹² such as silver nanoparticles¹³, silica¹⁴, or titanium dioxide nanoparticles¹⁵. In particular, carbon-based materials such as carbon nanotubes (CNTs), fullerenes (C60) and carbon nanofibers (CNF) have been shown to cause cellular aneuploidy^{10,16-18} and induce cell cycle arrest⁸⁻¹² in a dose- dependent manner¹⁰. Cellular exposure to CNTs, members of the fullerene family and allotropes of carbon¹⁹⁻²¹, known for their inherent mechanical²²⁻²⁶, electrical²⁷⁻³³, optical³⁴⁻³⁶ and thermal²⁴ properties that make them viable candidates for applications in engineering^{37,38} and bio-medical sectors^{35,39-44}, have been shown to lead to initiation of tumorigenesis through cell cycle disturbances^{8-12,17}. *In vitro* treatment with multi-walled carbon nanotubes (MWCNTs), comprised of multiple sheets of sp² hybridized carbon atoms rolled into a cylinder with concentric layers held together by secondary van der waals interactions^{19-21,24,30-32,45,46}, showed cell cycle changes to HeLa cells in culture⁹. A drop in the G1 phase and an increase in cells at the S/G2 phase border, indicative of a cell cycle check-point response, as well as MWCNTs impaired normal progression through cellular division were observed⁹. In agreement with these findings, Zhang et al., have showed that treatment with carboxylated MWCNTs led to up regulation of cyclin dependant kinase inhibitor 1 (p21), a known inhibitor of the cell cycle, following impaired bone morphogenic protein initiation (BMP)¹². Such damages caused by the MWCNTs treatment led to irreversible genetic mis-segregation^{1,4,47}. Further, studies have reported micronuclei formation via comet assay that confirmed breakage of DNA^{9,48-51} and pan-centromeric staining^{10,16,18}, which is indicative of breakage and/or disruption of the mitotic spindle apparatus. These findings combined with evidence of disturbed cell cycle regulation¹² illuminate part of the primary genotoxic pathways elicited following MWCNT exposure. However, of the scientific reports that are currently published there seems to be an incongruence of professional opinion in the area of CNT-induced genotoxicity⁵². Namely, these conflicting reports are mainly due to the lack of experimental consistency associated with the

CNT manufacturer, uncertain composition, purity and functionality, and exposure dose used in the experiments meant to elicit such toxic pathways.

Here, we report a detailed study on the potential molecular mechanisms underlying MWCNTs genotoxicity. In particular, we hypothesized that the changes in the cell cycle are associated with changes in the mRNA expression of integral molecular motor proteins, dynein and Eg5, involved in the cell division process. Further, we hypothesized that these changes are correlated to the cell progression through the cell cycle upon exposure to nanomaterials with different characteristics (i.e. physical and chemical properties). Human airway epithelial cells were used as a model system. In the cell, dynein and Eg5⁵³⁻⁶² control the dynamics of the spindle formation and help in even segregation of chromosomes^{63,64, 65}. Cytoplasmic dynein forms the dynein-dynactin complex^{54,61,66-69}; dynein is a minus end directed molecular motor, responsible for the transportation of intracellular organelles along the cytoplasmic microtubule network^{70,71}. Dynein localizes near the cell cortex⁶⁸, along the plus ends of growing microtubules near the nuclear envelope^{72,73} or at the centrosomes where it helps with centrosome separation^{62,69,74-76} and organization of radial microtubule arrays⁵⁴. Eg5 belongs to the kinesin-5 subclass of motor proteins and is a part of the Bim-C kinesin family⁷⁷⁻⁸¹. Eg5 is a slow moving, plus end directed homotetrameric motor protein⁷⁷⁻⁸¹ that localizes at the spindle poles⁵³ and along the interpolar microtubule network. Eg5 attaches to antiparallel microtubules, and provides directional force required to separate the spindle^{65,77-82}. Regulation of these molecular motors occurs in response to phosphorylation of cyclin dependant kinases (CDKs)^{53,55,83} i.e. cell cycle checkpoint regulators. Our data acquired using fluorescent activated cell sorting to quantify the DNA of the sorted cells, and real-time polymerase chain reaction to quantitatively measure the mRNA concentrations of the two molecular motors, provide a viable mechanical model to be associated with genotoxicity. Such expressional changes in integral molecular motor protein concentrations can result in mitotic catastrophe causing aneuploidy^{9,10,16,17}, and the initiation of tumorigenesis, genotoxicity and cancer progression.^{47,84,85}

Materials and Methods:

Multi-walled carbon nanotubes (MWNCTs) acids oxidation

Commercial multi-walled carbon nanotubes (MWNCTs) purchased from NanoLab Inc. (PD15L5-20, purity > 95%) were acid oxidized. MWCNTs were incubated in a concentrated sulfuric (96.4%, Fisher, USA) and nitric (69.5%, Fisher, USA) acids mixture with a volume ratio of 3:1 as previously described⁸⁶. The mixture was then placed on an ice bath in a sonicator for 1 or 3 h at a constant temperature of 23°C. Subsequently, the mixture was diluted in 2 L of deionized (Di) water and filtered through a GTTP 0.2 µm polycarbonate filter membrane (Fisher, USA). The procedure was repeated for several cycles to remove any acid traces. The MWCNTs isolated on the filter membrane were subsequently dried under the vacuum and stored at room temperature for future use.

MWCNTs physical and chemical characterization

Atomic force microscope (AFM) with Si tip (Asylum Research, 50-90 KHz AC240TS, USA) was used to quantify the length of pristine and acid oxidized MWCNTs. MWCNTs samples (1 and 3 h acids oxidized MWCNTs) were deposited on mica substrates (9.5 mm diameter, 0.15- 0.21 mm thickness, Electron Microscopy Sciences, USA) for analysis. Scans of 10 µm x 10 µm carried out in tapping mode were acquired. At least 30 individual MWCNTs from different images were collected for an average length distribution.

MWCNTs samples (both 1 and 3 h acids oxidized MWCNTs) were deposited on clean glass slides (Fisher Scientific, USA) for chemical structure investigation. Raman spectroscopy was performed using a Renishaw InVia Raman Spectrometer (CL532-100, 100 mW, USA) operating at room temperature and under an excitation wavelength of 514.5 nm. The radiation was focused through a 20 X objective for an area of < 0.01 mm². Further, low energy (i.e., < 0.5 mV) and a short exposure time of only 10 sec were employed to avoid unexpected heating effects of the samples. The scanned ranges were in between 100–3200 cm⁻¹.

Energy dispersive R-ray spectroscopy (EDX) was employed for quantitative elemental analysis of the acid oxidized MWCNTs as previously described⁸⁶. Briefly, the samples (1 mg/ml in water, either 1 or 3h acids oxidized MWCNTs) were placed on a silica substrate and dried

under vacuum. A Hitachi S-4700 Field Emission Scanning Electron Microscope (USA) with an S-4700 detector, integrating secondary (SE) and backscattered (BSE) electron detection (in a single unit), was used to carry out the analysis at 20 kV. Data is represented as elemental weight percent.

MWCNTs sample solubility (either 1 or 3 h acids oxidized MWCNTs) was evaluated using a standard dispersity test^{86,87}. Briefly, 3 mg of MWCNTs were diluted in 1mL of either DI water, phosphate buffered saline (PBS, Invitrogen, USA) or Dulbecco's Modified Eagle Media (DMEM, Invitrogen, USA) with 10% Fetal Bovine Serum (FBS, HyClone, USA) and 1% antimycotic and antibiotic supplement (Anti/Anti, GIBCO, USA). The over-concentrated MWCNTs suspension was centrifuged for 5 min at 3000 rpm; subsequently, 0.8 mL supernatant was removed and filtered through a 0.2 μ m GTTP filter membrane. The solubility of the MWCNTs was then calculated based on the difference between initial starting weight and final weight of the dispersed sample.

Cell culture and treatment

Immortalized human bronchial respiratory epithelial cells (BEAS-2B, ATCC, USA) of passage < 10 were used to examine the potential toxicological effects of MWCNTs. Cells were cultured in DMEM media supplemented with 10% FBS, 1% antimycotic and antibiotic at 37°C, and 5% CO₂ flow.

For treatment, BEAS-2B cells were seeded in T-25 flasks (Corning, USA) at a cell density of 2×10^6 cells in 3 mL of culture media and allowed to achieve 70-85% confluency. Cell confluency and health was assessed using light microscopy (Olympus Optical Co., LTD, Japan) through a 20X objective, in bright field. Three independent replicates were exposed for 24 h to 0.24 μ g/mL MWCNTs (either 1 or 3h acids oxidized MWCNTs) diluted and sonicated in media for 1 min under 10 sec pulse intervals delivered by a probe sonicator (Ultrasonic Processor 130W, 20kHz, Sonics & Materials, Inc., USA). Following 24 h exposure, cells were recovered with fresh media and/or isolated or allowed to continue in culture for subsequent studies.

Fluorescent Assisted Cell Sorting (FACs)

Following 24 h treatment, treated and control cells (not exposed to MWCNTs) were removed from T-25 flasks using 0.25% trypsin digestion (GIBCO, USA), pelleted by centrifugation at 1000 rpm for 6 min, resuspended in 1mL PBS, spun again at 1000 rpm for 6 min and then resuspended in 200 μ L PBS. The resuspended cells were subsequently fixed in 2 mL of ice cold 70% biological grade ethanol (Sigma, USA) and stored at -20°C.

For Fluorescent Assisted Cell Sorting (FACs) analysis, fixed cells were pelleted at 1000 rpm for 6 min. The ethanol was then decanted and cells were resuspended in 2 mL of PBS, incubated at room temperature for 60 sec, pelleted at 1000 rpm for 6 min, resuspended in 100 μ L of 0.2% Tween 20 (Sigma, USA) in PBS, and incubated for 15 min at 37°C. Subsequently, 100 μ L of PBSAz (PBS-Sodium Azide, Sigma USA, i.e. wash buffer comprised of PBS+0.5%BSA+0.02% Sodium Azide) was added to the cell suspension. Cells were again pelleted at 1000 rpm for 6 min, resuspended in 10 μ L of RNaseA –PBS solution (180 μ g/mL, Invitrogen, USA) per 5×10^5 - 1×10^6 cells and incubated for 15 min. Finally, 30 μ L of propidium iodide stain (final concentration 75 μ g/mL, Sigma, USA) per 5×10^5 - 1×10^6 cells was added to the cell suspension followed by cell incubation on ice for 15-30 min.

FACS analyses were carried out using a FACsCalibur (BectonDickson, USA). 50,000 events were counted; all data was normalized to 30,000 events using FCS Express 4 Flow Cytometry software (De Novo Software, USA). Results are presented as % of diploid cells in the G1, S or G2 phases.

RNA isolation

Cells exposed to MWCNTs for 24 h or the controls were lysed. Specifically, 1 mL RLT lysate buffer (included in RNeasy Mini Kit, Qiagen, USA) was mixed with 10 μ L β -Me (Sigma, USA), and 700 μ L of this lysate was added per flask. Flasks were rocked briefly by hand to ensure efficient lysate buffer distribution and incubated for 10 min. Flasks were next scraped with a cell scraper and the 700 μ L volume was removed using a pipette. Using RNeasy Qiashredder columns (Qiagen, USA) and RNeasy Mini kit (Qiagen, USA), RNA was isolated and purified according to manufacturer specifications. Also, a column DNase treatment was performed where 80 μ L of DNase I treatment per sample, i.e. 10 μ L of DNase I stock in 70 μ L of

Buffer RDD (DNase I treatment kit, Qiagen, USA), was applied to the columns and incubated for 20 min at room temperature. Finally, RNA was eluted to a final elution volume of 30 μ L with nuclease free water. Microcentrifuge tubes containing the eluted total RNA were placed on ice. Absorbance was measured using the NanoDrop 2000 (THERMO Scientific, USA) blanked to nuclease free water. Absorbance readings were done in duplicates and averages were taken for cDNA synthesis calculations. RNA samples were stored at -80°C.

RNA Quality Assessment

RNA 260/230 absorbance readings of > 1.75 and 260/280 absorbance readings of > 1.9 and < 2.2 were indicative of high integrity and quality samples⁸⁸. Further quantitative analysis of the isolated RNA was carried out using the Bioanalyzer 2100 Agilent system (Agilent Technologies, Inc., USA) and software (Agilent Technologies, Inc., USA). Briefly, aliquots of isolated RNA were added to patented reagents, following manufacturer specifications; next, they were placed into the Agilent Eukaryote Total RNA Nano microchips and analyzed using the Bioanalyzer 2100. An RIN number > 7 indicated high integrity RNA that was then used for cDNA synthesis and RT-PCR⁸⁹.

cDNA synthesis

cDNA synthesis was performed in a 20 μ L reaction volume using the manufacturer directions for First Strand Synthesis System for RT-PCR (Invitrogen, USA). Oligo dT was used as a primer to achieve more consistent RT-PCR products⁹⁰ and to provide more specific hybridization of the 3' poly(A) tails. Briefly, RNA template (1.5 μ g per sample) and DEPC-water were added to Primer (1 μ L per sample) and dNTPs (1 μ L per sample) for a total volume of 10 μ L in 1.5 mL microcentrifuge tubes. Subsequently, the tubes were incubated in a 65°C water bath for 5 min; upon incubation the tubes were removed and immediately placed on ice. Next, 10XRT buffer (2 μ L per sample), 25 mM MgCl₂ (4 μ L per sample), 0.1 M dithiothreitol (DTT) (2 μ L per sample), RNaseOUT (40 U/ μ L, 1 μ L per sample) and Superscript III RT (200 U/ μ L, 1 μ L per sample) were added according to manufacturer specifications for a 20 μ L reaction volume, and the mixture was incubated in a 50°C water bath for 1 h. All reagents were purchased from Invitrogen USA unless otherwise specified. Following the 1 h incubation, amplification was stopped by moving the tubes to a heating block at 85°C for 5 min, and then

placing tubes on ice. Finally, 1 µL of RNase H was added to each tube and incubated at 37°C for 20 min. Samples were diluted five times in nuclease free water and stored at -20°C.

Standard Curve Method for Real time-polymerase chain reaction

Real time-polymerase chain reaction (RT-PCR) was performed using the Applied Biosystems 7500 and 7900HT cyclers (Applied Biosystems, USA). Taqman RT-PCR gene expression assays (Applied Biosystems, USA) were used, to ensure high quality detection. Briefly, cDNA was thawed on ice. Also placed on ice were Taqman Universal Master Mix and Taqman Gene Expression Assay Primers (Dynein-Hs00322286_m1 and Eg5-Hs0189698_m1, Applied Biosystems, USA) and nuclease free water. Components were added according to manufacturer specifications. Briefly, 5 µL of cDNA, 1 µL of primer, and 10 µL of Taqman Universal Master Mix were added per well per sample for a reaction volume per well of 20 µL adjusted with nuclease free water. Standard curves were established for each primer using pooled serially diluted PBS control cDNA. All reactions were carried out on a 96well reaction plate (Applied Biosystems, USA). The reaction plate was covered with a transparent and adherent plate film (Applied Biosystems, USA) and centrifuged at 1200 rpm for 3 min prior running the experiment. For both the 7500 and 7900 cycler systems, relative quantifications were performed. Primers were selected and plate layout was designed as per software (Applied Biosystems, USA) requirements. The run was carried out under default settings adjusting for a 20 µL reaction volume for a total of 1 ½ h. Auto CT was selected and data was analyzed using the RQ Manager Software package (Applied Biosystems, USA).

Statistical analysis

Data were analyzed using SAS software (North Carolina, USA), employing Mixed Procedure. F-tests for fixed effects and tables for least squared means were generated and analyzed for both FACs and RT-PCR procedures. Differences were evaluated using the Tukey-Kramer procedure, and p-values of 0.05 were considered significant.

Results:

Multi-walled carbon nanotube physical and chemical characterization

In the present study, two carboxylated multi-walled carbon nanotubes (MWCNTs) were prepared using oxidation with concentrated nitric and sulfuric acids for 1 and 3 h respectively. The resulting average length of the MWCNTs was determined using atomic force microscopy (AFM) and tapping mode. As shown in Figure 1A, the analyses revealed an average length of 903 ± 368 nm and 616 ± 344 nm for the 1 and 3 h acids oxidized MWCNTs respectively. The shift towards shorter lengths observed for the 3 h acids oxidized MWCNTs is in agreement with previous reports that showed that acids mixture reacts with the C-C band of the nanotube graphene sheets and induces oxidization and functionalization of nanotubes at defects site along their side walls, thus generating shorter nanotubes^{86,91}.

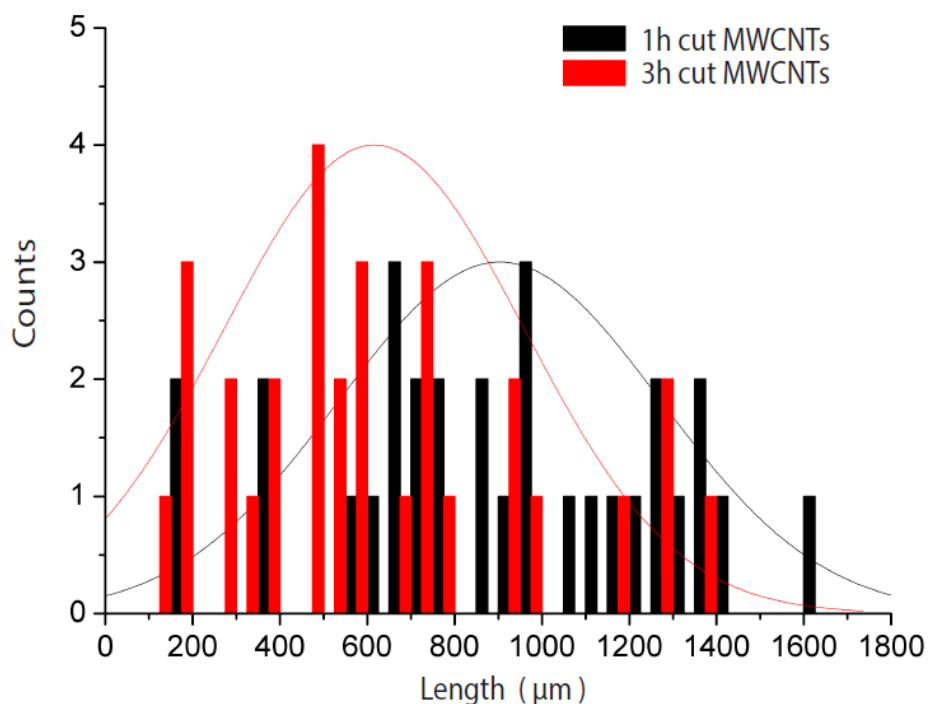


Figure 1A: Characterization of the multi-walled carbon nanotubes (MWCNTs). A) Histogram of Average Length distribution for (a) 1h acids oxidized MWCNTs (black) 903 ± 368 nm, and (b) 3h acids oxidized MWCNTs (red) 616 ± 344 nm, identified by tapping mode Atomic Force Microscopy (AFM).

Table 1: Raman spectra analyses of the 1 and 3 h acids oxidized MWCNTs.

Sample	D band position (cm ⁻¹)	G band position (cm ⁻¹)	I_D/I_G Intensity ratio
1hr acid-treated MWCNTs	1348	1585	0.788
3hr acid-treated MWCNTs	1349	1589	0.804

Table 2: Energy dispersive X-ray analyses of 1 and 3 h acid oxidized MWCNTs.

Element %	1h cut MWCNTs	3h cut MWCNTs
C	95.29	95.96
O	3.07	3.45
Fe	1.64	0.59

To determine the chemical properties of the acids oxidized MWCNTs, Raman and energy dispersive X-ray spectroscopy (EDX) analyses were performed. Raman spectrum of the 1 and 3 h acids oxidized MWCNTs are shown in Figure 1B while the peak characteristics are shown in Table 1. The D peak (1350 cm⁻¹) relates to the presence of amorphous carbon on the MWCNTs sidewall and the G peak (1590 cm⁻¹) is an indicator of high degree and well-structured crystal carbon based species. The two peaks were shifted towards higher frequencies for the 3 h acids oxidized MWCNTs when compared to 1 h sample. Further, the D and G peaks relative intensity ratio I_D/I_G , which indicates the degree of functionalization of the MWCNTs and is usually associated with the presence of free carboxyl acidic groups created at the nanotube wall interface, was 0.788 for 1 h and 0.804 for 3h acids oxidized tubes respectively. This suggested that the increased number of carboxyl groups on the nanotube surface was proportional to longer

acid treatment time ⁹². The shifts in the additional peaks i.e. G' peak at 2670 cm⁻¹ and a peak at 2920 cm⁻¹ were associated with washing away the catalyst metal particles initially present in the pristine MWCNTs, thereby increasing electron-accepting groups. EDX analysis further confirmed that acid oxidation removed the metal catalysts leading to the carboxyl functional groups formation. The elemental compositions of the MWCNTs following 1 and 3h oxidation are shown in Table 2. A decrease of the iron catalyst was observed for the 3 h acid oxidized sample when compared to the 1 h sample indicating that longer cutting times removed the catalyst to a larger extent. Furthermore, longer cutting times led to an increase of the oxygen content further confirming oxygen incorporation (i.e. formation of free carboxyl groups) on the nanotube side wall upon the longer oxidation time.

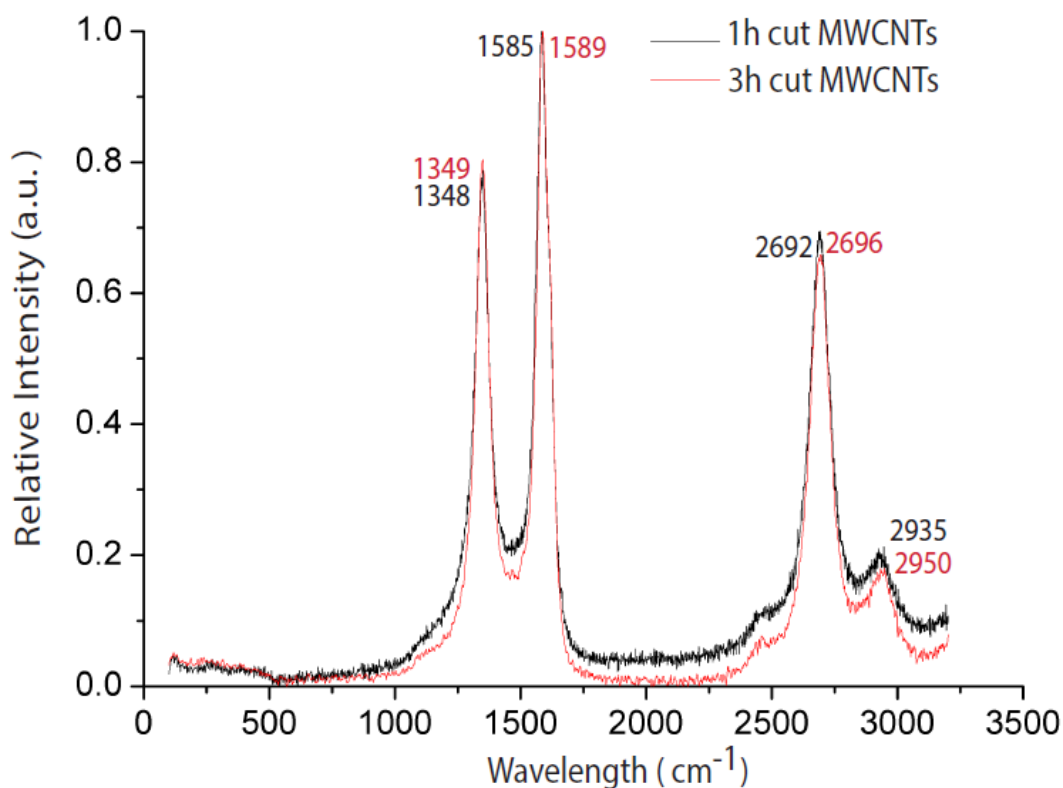


Figure 1B: Characterization of the multi-walled carbon nanotubes (MWCNTs). B) Raman Spectra of 1h acids oxidized MWCNTs (black), and 3 h acids oxidized MWCNTs (red).

Dispersity of the 1 and 3 h acids oxidized samples was also evaluated. Highly dispersed nanotubes will ensure uniform treatment of the cellular systems and thus reduce permeability

limitations while increase their net flux⁹³ across the cell membrane. Our studies and others have shown that solubility of the MWCNTs samples can be attributed to the combined effects of generation of carboxylate anions derived by the de-protonation of surface reactive carboxylic groups and the stacking π - π interactions between the MWCNTs surface and the amino acid⁹⁴, and protein⁹⁵ components in the cell culture media. Specifically, the dispersity of the MWCNTs samples (both 1 and 3 h acids oxidized) was evaluated in different dispersing agents (DI water (pH 6.25), PBS (pH 7) and DMEM including 10% FBS and 1% Anti/Anti media, or culture media). The results are shown in Figure 1C and indicated that the dispersity of the MWCNTs (both 1 and 3 h acids oxidized) in culture media was significantly improved when compared to the dispersity of the MWCNTs in the other agents. Further, the dispersity of the 3 h sample was higher than the one of the 1 h sample. The increase in solubility of MWCNTs samples after longer acids oxidation is correlated with the increase in the number of carboxylic acidic groups upon sample treatment as indicated both by the Raman and EDX analysis. The increased solubility observed for the MWCNT samples in PBS compared to Di water can be attributed to the greater de-protonation of the carboxyl acid groups at relative higher pH values⁹⁶.

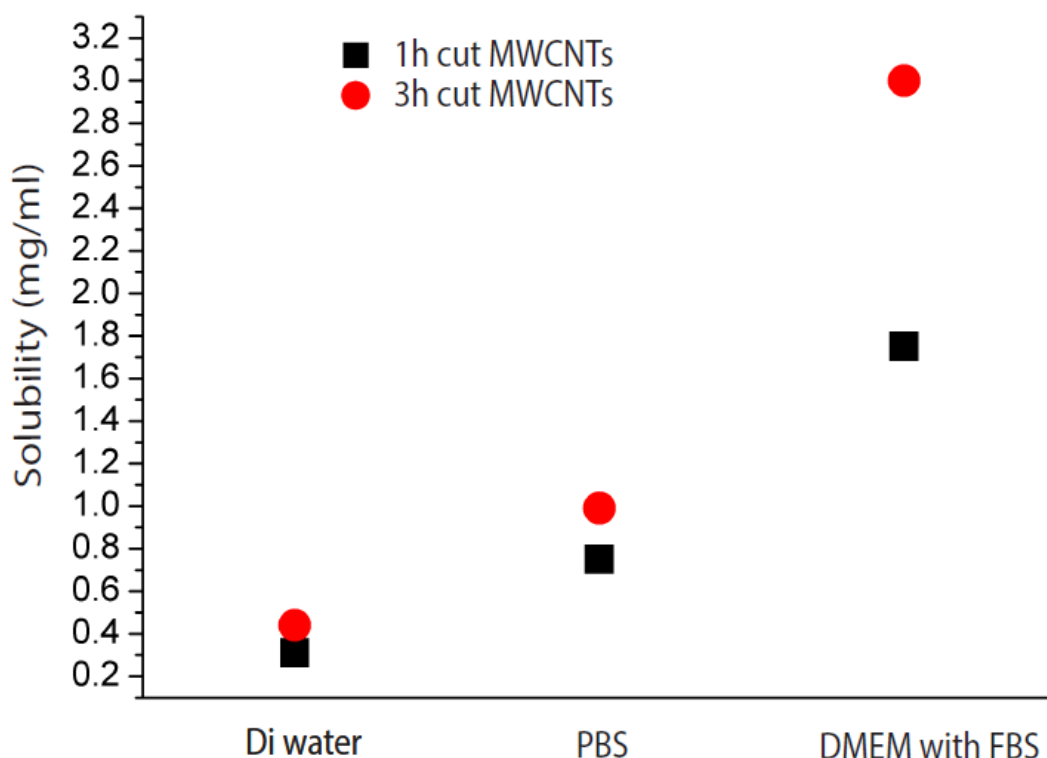


Figure 1C: Characterization of the multi-walled carbon nanotubes (MWCNTs). C) Dispersity of 1 and 3 h acids oxidized MWCNTs in water, PBS and media. The dispersity of 3 h was considerably improved relative with 1 h acids oxidized MWCNTs.

Cell Cycle Analysis

Physically and chemically characterized MWCNTs were used for treatment of immortalized human bronchial respiratory epithelial (BEAS-2B) cells (Figure 2A). Currently, occupational exposure guidelines indicated for nanomaterials (including MWCNTs) provide a recommended exposure limit (REL) of $7 \mu\text{g}/\text{m}^3$ ⁹⁷. Previous studies using *in vitro* models, used CNT dosages in excess, with impurities and defects^{18,48-51,98}, and/or have been calculated assuming linearity from *in vivo* studies based on the surface area of the lung of the *in vivo* model organism^{9,10,99}. In our studies, we treated cells with $0.24 \mu\text{g}/\text{mL}$ for 24 h. This dosage was based on *in vivo* studies carried out at an exposure dosage of $20 \mu\text{g}/\text{mouse}$ ¹⁰⁰, equivalent to 40 h of exposure for a 20 week duration at the OSHA particle exposure limit (PEL) of $5\text{mg}/\text{m}^3$ ¹⁰¹.

10,100,101

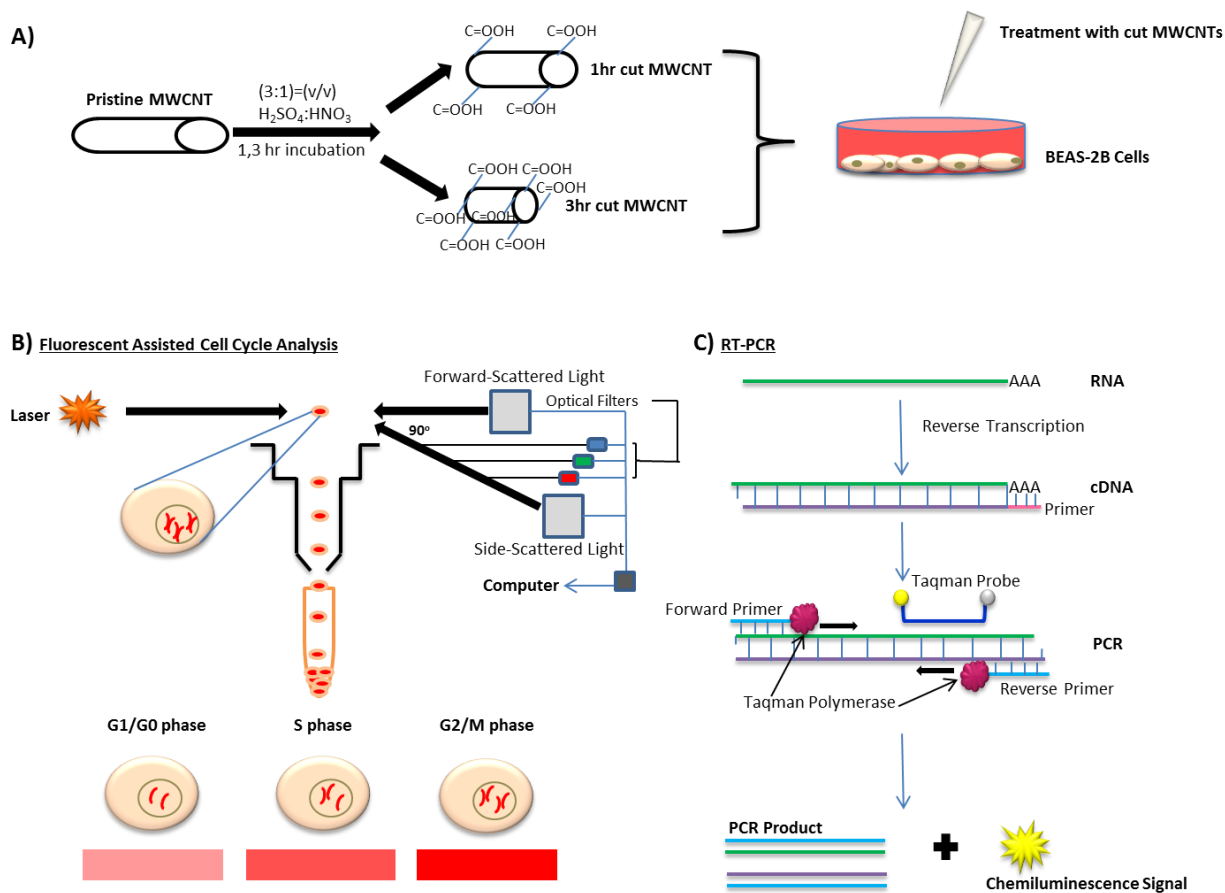


Figure 2: Cell Treatment and Analysis Summary Diagram: Procedural overview of the experiments: **A)** MWCNTs were acid-treated for 1 and 3 h and to lead to carboxyl functionalized nanotubes with different lengths and surface properties. Thus characterized MWCNTs are used to treat confluent BEAS-2B cells in culture dishes. **B)** Following cellular exposure to either 1 or 3 h acid oxidized MWCNTs for 24 h, cells were stained with propidium iodide and subjected to cell cycle analysis by flow cytometry for finding of DNA content abnormalities. **C)** Following cellular exposure to either 1 or 3 h acid oxidized MWCNTs for 24 h, real-time polymerase chain reactions (RT-PCR) was performed to identify any changes in the expression pathways of the key molecular players of the cell division.

Table 3: Summary Table of Flow Cytometry Cell Cycle Analysis for G1 and S phases: The analysis shows that 3 h acids oxidized MWCNTs treatment resulted in significant changes (p-value < 0.05) for both G1 and S phase, when compared to the changes induced by the 1 h acids oxidized MWCNT or the PBS controls.

G1 Phase

Treatment	% Diploid Cells at 0 h	% Diploid Cells at 24 h	% Diploid Cells at 48hr
PBS Control	53.2	45.9	49.7
1h acids oxidized MWCNTs	54.9	39.7	42.9
3h acid oxidized MWCNTs	49.0	49.4	27.4*

S Phase

Treatment	% Diploid Cells at 0 h	% Diploid Cells at 24 h	% Diploid Cells at 48hr
PBS Control	37.3	39.5	36.48
1h acids oxidized MWCNTs	35.4	46.7	48.5
3h acids oxidized MWCNTs	40.3	39.5	63.9*

To address whether cell cycle was affected following cellular exposure to MWCNTs (either 1 or 3 h acids oxidized), fluorescent activated cell sorting (FACS) analyses was performed (Figure 2B). Previous studies have reported that the cell cycle arrests upon nanomaterial exposure due to direct interactions with both the mitotic spindle ^{9,10}, namely the cytoskeletal filament microtubule and with the DNA ^{16,49}. These interactions have been linked to cell proliferation blockage, mitotic spindle disturbances quantified by centrosome fragmentation ¹⁶ and DNA clastogenic affects ¹⁶. For our research, cells were incubated with MWCNTs for 24 h, subsequently isolated at 0, 24 and 48 h from culture and stained with propidium iodide for DNA labeling. Following isolation and staining, analyses were performed using the FACS Calibur and the data was normalized to 30,000 events.

The results are shown in Figure 3; the first peak (red) indicates cells in G1 phase, the second peak (blue) indicates cells in S phase and the third peak (green) indicates cells in G2 phase, respectively. Following exposure to 3 h acids oxidized MWCNTs cells moved out of G1

phase into S phase, with no significant change in G2 phase when compared to the controls. Two-way ANOVA showed that there was a significant difference (p -value < 0.05) for both G1 and S phases in cells exposed to 3 h acids oxidized MWCNTs isolated at 48 h when compared to PBS controls or 1h acids oxidized MWCNT treatment. Specifically, the 3 h acids oxidized MWCNTs treatment increased the percent of diploid cells in S phase to (mean % = 63.9, $p < 0.05$) when compared to PBS controls (mean % = 36.48, $p > 0.05$) and 1h acids oxidized MWCNTs (mean % = 48.5, $p > 0.05$) treatment. For the cells in G1 phase the exact opposite was observed. The 3 h acids oxidized MWCNTs treatment decreased the percent of diploid cells to (mean % = 27.4, $p < 0.05$) when compared to PBS control (mean % = 49.7, $p > 0.05$) and 1 h acids oxidized MWCNTs (mean % = 42.9, $p > 0.05$) treatment. Finally, neither treatment with 1 or 3 h acids oxidized MWCNTs affected cells in G2 phase when compared to the control PBS. The observed shift of the cells incubated with 3 h acids oxidized MWCNTs from G1 to S phase can be associated with an overall decrease in cellular division over time in response to the treatment^{9,10}. The cell treated with 1 h acids oxidized MWCNTs did not show a significant loss of cell division over time in the same time frame when compared to the controls.

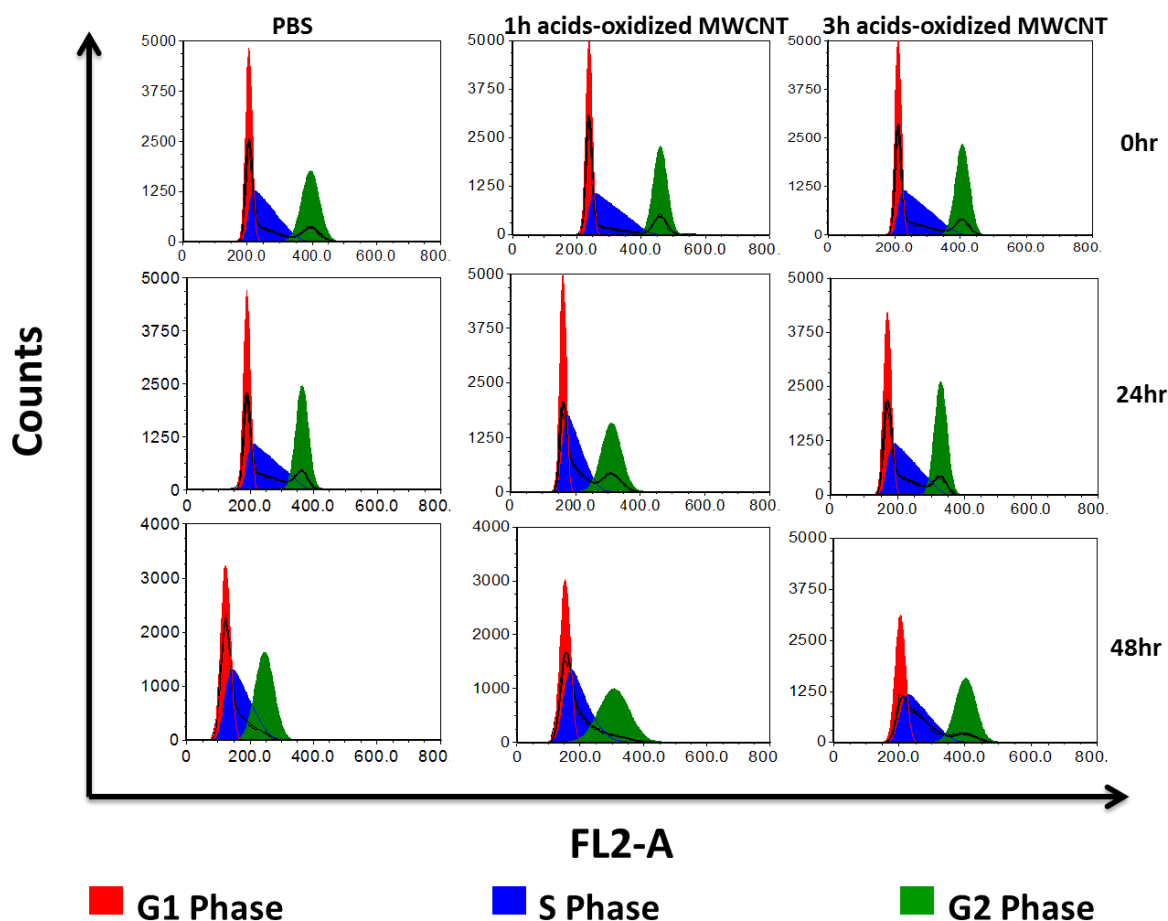


Figure 3: Graph of Flow Cytometry Cell Cycle Analysis: Cells stained with propidium iodide were read using the FACsCalibur flow cytometer, analyzed for their DNA content and sorted into G1 (red), S (blue), or G2 (green) phase, timed isolation (0, 24, 48 h) and treatment (1 or 3 h acids-oxidized MWCNTs) respectively. For the 3h acid-oxidized MWCNT group a clear decrease in G1 (red) phase and subsequent increase in S (blue) phase is shown for the 48 h time point as compared to the 1h acid-oxidized MWCNT or PBS control groups.

Dynein and Eg5 mRNA Expression

Collective studies have shown that cell cycle arrest results in the destruction and expression of several regulatory proteins from down regulation of cyclin dependant kinases (CDKs) ¹ to up regulation of inhibitory p21 ¹⁰² and p27 CDKs ¹⁰³. Further, studies have reported that interaction of CNTs with cellular elements, microtubules ⁹, centrosomes ^{10,16,18}, and DNA ^{16,18,49}, and have resulted in changes in cellular elasticity ¹⁰⁴ that coordinated with changes in the

cell cycle^{9,10}, as an early indicator of tumorigenesis¹. Based on these observations, we hypothesized that the observed cell cycle changes (Figure 3), could lead to changes in integral motor proteins associated within the cellular division process. In particular, we focused on two of the mitotic molecular motors, dynein and Eg5. Dynein is a minus end molecular motor that coordinates centrosome separation for successful bipolar spindle assembly^{61,66,68,75,76,105}, while Eg5 is a plus end molecular motor that provides anti-parallel sliding forces necessary to evenly segregate genetic material into two daughter cells^{53,56,57,77,82,106}. To address whether the integral mitotic molecular motor protein expression was affected upon cellular exposure to MWCNTs (both 1 and 3 h acids oxidized), RT-PCR was performed (Figure 2C). Briefly, RNA was isolated from cells treated with both 1 and 3 h acid oxidized MWCNTs and from control cells. High degree of RNA integrity was reversed transcribed into cDNA and RT-PCR was performed to quantify mRNA expression of the two molecular motors across 0, 24, 48 and 72 h.

The dynein and Eg5 mRNA expressions upon MWCNTs acids oxidized treatment are shown in Figure 4. Both dynein and Eg5 expressions were significantly (p -value < 0.05) reduced after 72 h post treatment when compared to controls. Interestingly however, the reduction in Eg5 expression in cells exposed to 3 h acids oxidized MWCNTs was observed after 48 h when compared to 1 h acids oxidized samples or control. Results shown in Figure 4 indicate that for dynein expression treatment with 1 or 3 h acids oxidized MWCNTs lead to roughly a 50% reduction (arbitrary units) in mRNA expression at 72 h, while for Eg5, treatment with 1 or 3 h acids-oxidized MWCNTs lead to roughly a 37.5% reduction (arbitrary units) in mRNA expression at 72 h and roughly a 15% reduction (arbitrary units) at 48 h for 3 h acids oxidized treatment only.

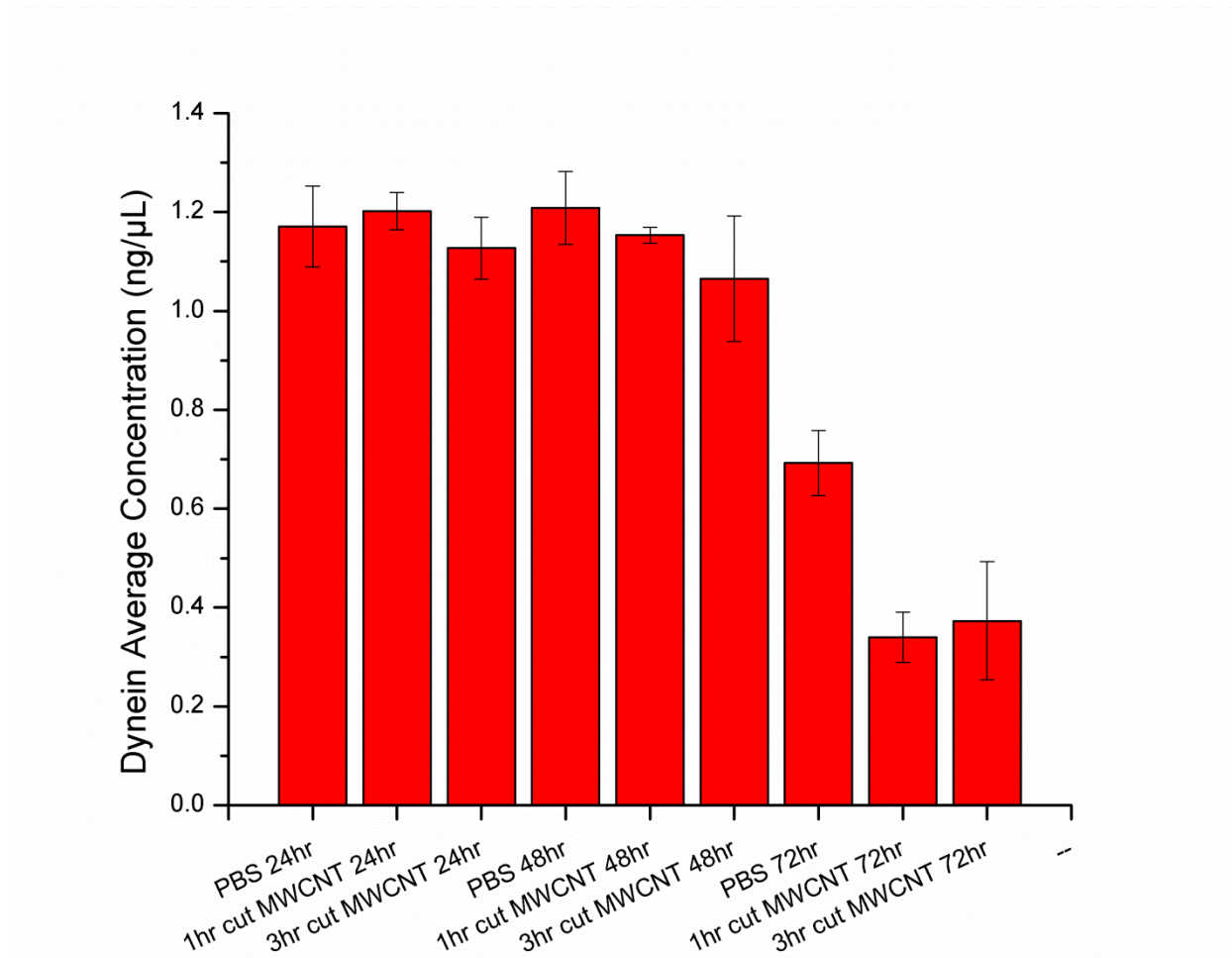


Figure 4A: Changes in expression levels of key proteins involved in the cell division. A)

Graph of Dynein mRNA Expression: Cellular treatment for 24 h with both 1 and 3 h acids oxidized MWCNTs led to significant (p -value < 0.05) decreases in mRNA expression concentrations (ng/μL) as compared to PBS controls at 72 h.

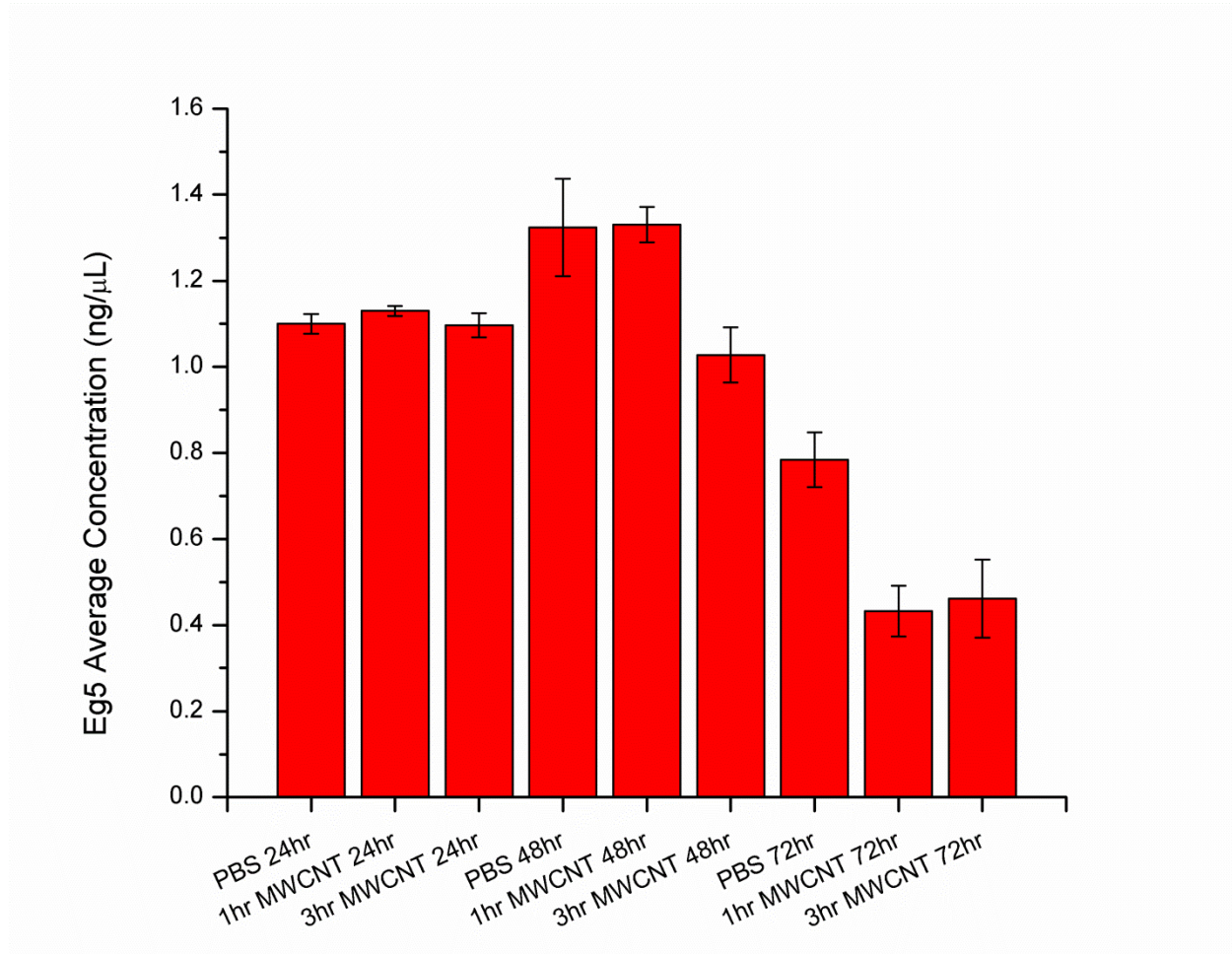


Figure 4B: Changes in expression levels of key proteins involved in the cell division. B)

Graph of Eg5 mRNA Expression: Cellular treatment for 24 h with both 1 and 3 h acids oxidized MWCNTs led to significant (p -value < 0.05) decreases in mRNA expression concentrations (ng/μL) as compared to PBS controls at 72 h. Additionally treatment with 3 h acids oxidized MWCNTs led to significant (p -value < 0.05) decrease in mRNA expression concentrations (ng/μL) at 48 h when compared to 1 h acids treated MWCNTs and PBS controls.

Discussion:

Cell cycle consists of a comprehensive regulatory network of external and internal signals also influenced by environmental factors to coordinate cell division that ensures growth, differentiation and tissue formation ^{6,107}. As cells progress through their cell cycle, distinct

proteins are being synthesized or degraded to ensure cycle-dependent turnover ^{6,108}. Such complex synthesis or degradation processes are dependent on distinct biochemical activities that take place inside the cells to ensure continuous regulation of RNA and protein levels maintenance ^{6,109}. Defects in the cell cycle as well as defects in the complex synthesis of the key protein players in cell cycle progression may lead to chromosome alterations, aneuploidy and ultimately cancer ^{1,4}. Our study aimed to evaluate the effects of acids oxidized MWCNTs treatment on the lung epithelial cell cycle and associated molecular motor proteins players in cell division.

We showed that the effects on the cell cycle were dependent on the MWCNTs properties (both chemical and physical). For instance, our results showed that following exposure for 24 h to 3 h acids oxidized MWCNTs, cells moved out of G1 phase (41% decrease in G1 phase) and were subsequently arrested in the S phase (33.5 % increase in diploid cells in S phase) of the cell cycle at 48 h, with no apparent changes observed for the 1 h acids oxidized MWCNTs in the same time interval relative to the control (not exposed cells). Additionally, we showed no observable % change in diploid cells following either 1 or 3 h acids oxidized MWCNTs treatment at 24 h, suggesting a maximal response time of 48 h, also dependent on the MWCNTs properties (both chemical and physical). These findings are presumably an indicator of cell proliferation blockage ^{9,10,17}, with cells responding to treatment by arresting the cell cycle in an attempt to recover and/or repair cellular elements ¹ probably affected by exposure to MWCNTs and their physical and chemical properties. Our studies add to previous observations of cell cycle changes upon MWCNTs ⁹ exposure. For instance, Zhang et al., have reported that treatment with carboxylated MWCNTs mediates over-expression of cyclin-dependant kinase inhibitor 1 (p21), a known inhibitor of CDK1 & 2, independent of tumor protein 53 (p53) protein expression, via impaired bone morphogenic protein (BMP) signaling, suggesting that p21 alone, and not with p53 cooperation mediates cell cycle arrest in response to treatment with functionalized MWCNTs ^{11,12}. Further, Nam et al., found after treating normal rat kidney epithelial cells with sodium dodecyl sulfate (SDS) functionalized SWCNTs a significant reduction in CDK2 and CDK6 cell cycle proteins, reduced phosphorylated-retinoblastoma (pRB) and lowered cell viability were observed, while at the same time p53 and p21 expression increased and growth arrest at the G0/G1 phase border was observed ⁸.

In the present investigations, we also quantified the levels of expression for dynein and Eg5, key molecular motors involved in the cellular division process, and how such levels were affected by exposure to acids oxidized MWCNTs. In particular, dynein and Eg5 mRNA concentrations were reduced in a time and MWCNT functionalization-dependent manner by 50 and 37.5 % respectively, following exposure to both 1 and 3 h acids oxidized MWCNTs at 72 h, when compared to the PBS control. Additionally, following treatment with 3 h acids oxidized MWCNTs only, Eg5 mRNA concentration was down-regulated by 15% at 48 h. With respect to the differences recorded between exposure to 1 and 3 h acids oxidized MWCNTs, the distinct response in the cell cycle and protein expression could be attributed to the different characteristics of the MWCNTs. In particular, with an increase in the hydrophilicity and shorter nanotubes present at the cell incubation site, the ability of those nanotubes to be taken up and cross the biological barrier of the cell membrane is higher than for the longer and more hydrophobic nanotubes^{51,98,110-112}.

Our results showed that following exposure for 24 h to 3 h acids oxidized MWCNTs, cells moved out of G1 phase (41% decrease in G1 phase) and were subsequently arrested in the S phase (33.5 % increase in diploid cells in S phase) of the cell cycle at 48 h. Based on previous studies it is expected that dynein is normally up-regulated in the S phase of the cell cycle, adding to centrosome duplication and centrosomal segregation^{62,69,74-76}, and the organization of microtubule arrays⁵⁴ of the mitotic spindle. However, no changes in the expression level of dynein were observed in our studies after 48 h exposure to acids-oxidized MWCNTs. Further, instead of an up-regulation of the motor upon 72 h, the concentration of dynein was reduced to 50% when compared to the control. Similarly, Eg5 is normally up-regulated in late G2 phase and is required throughout the mitotic process^{56,57,60,62,113}. However, our studies showed that there was a 15% at 48 h and a 37.5 % at 72 h reduction following exposure to both 1 and 3 h acids oxidized MWCNTs, when compared to the PBS control. These are presumably due to the defects that exposure to MWCNTs introduced to the cellular cycle and the cell division apparatus. In particular, previous studies have shown that upon 24 h exposure of the cells to MWCNTs, nanotubes localize at the cell nucleus and they change the biomechanics of the cells¹⁰⁴. MWCNTs could thus lead the reorganization of the cytoskeletal and disruption of the mitotic spindle¹¹⁴. Further, upon their integration with cellular microtubules, a biohybrid could be formed¹¹⁵ to induce errors in chromosome numbers¹¹⁶ and thus point to cell transformation in

cancer cells¹¹⁷. As the cells are being arrested in the pre-G₂ phases of the cell cycle, the cell could not progress through a normal division process and thus through a normal cell cycle, to be able ensure the quality and the control associated with reliability in cellular DNA replication or in the maintenance of the cell cycle and responsible proteins involved with cell cycle progression. The DNA damage that could have resulted upon association with the MWCNTs^{16,18,48,49,51,98}, as well as the defects in the spindle formation⁹ may modulate epithelial cell responses germane to genotoxic stress⁵, cell death^{6,7}, or initiation of cancer^{1,4}.

The combination of the cell cycle and molecular motors analyses might also suggest an overall destabilization of the mitotic spindle assembly as a result of treatment with MWCNTs (Figure 5). Generally, there has to be a subtle balance within the cell cycle between increasing molecular motor activity/concentration⁷³, and the greater positive and negative cytoplasmic damping forces that act upon them⁷³, while at the same time maintaining microtubule and spindle integrity⁵⁴ relative to critical timings of the cell cycle (Figure 5A). Our data showed a disruption in the expression of these important mitotic players at times critical to their function that could presumably lead to abnormalities in the spindle assembly (Figure 5B). For instance, at 48 h, a critical doubling time for BEAS-2B cells¹¹⁸ when Eg5 activity is required for spindle formation, cells treated with 3 h acids oxidized MWCNTs lack full expression of Eg5, i.e. a reduction of 15%. Thus, the spatial relationship of opposing-forces generated between the mitotic molecular motor proteins, dynein and Eg5 will not be balanced to promote a normal spindle formation leading to monopolar spindle formation. Previous research showed that when an Eg5 small molecule inhibitor, monastrol was administered, or siRNA Eg5 expression knockdown occurred, cell cycle arrest^{57,60,62,119,120} and monopolar spindle formation^{62,119,120} resulted. Further, depending on when Eg5 is inhibited during the mitotic process, spindle assembly might not occur at all. Ferenz et al., have reported for instance that if centrosomes are $\leq 5.5\mu\text{m}$ apart, and microtubule arrays were not well established at the time Eg5 was inhibited, dynein was unable to functionally interact with the underdeveloped microtubule region, in an act to rescue centrosome separation and provide antiparallel sliding motion, hence resulting to monopolar spindles formation⁵⁸. Additionally, this study suggested a novel role for dynein⁵⁸, also supported by recent research that showed that spindle assembly requires the binding of the microtubule to the p150 subunit of dynactin¹²¹ at the CLIP-170 region where it then binds to dynein and antagonizes Eg5 movement⁶⁹.

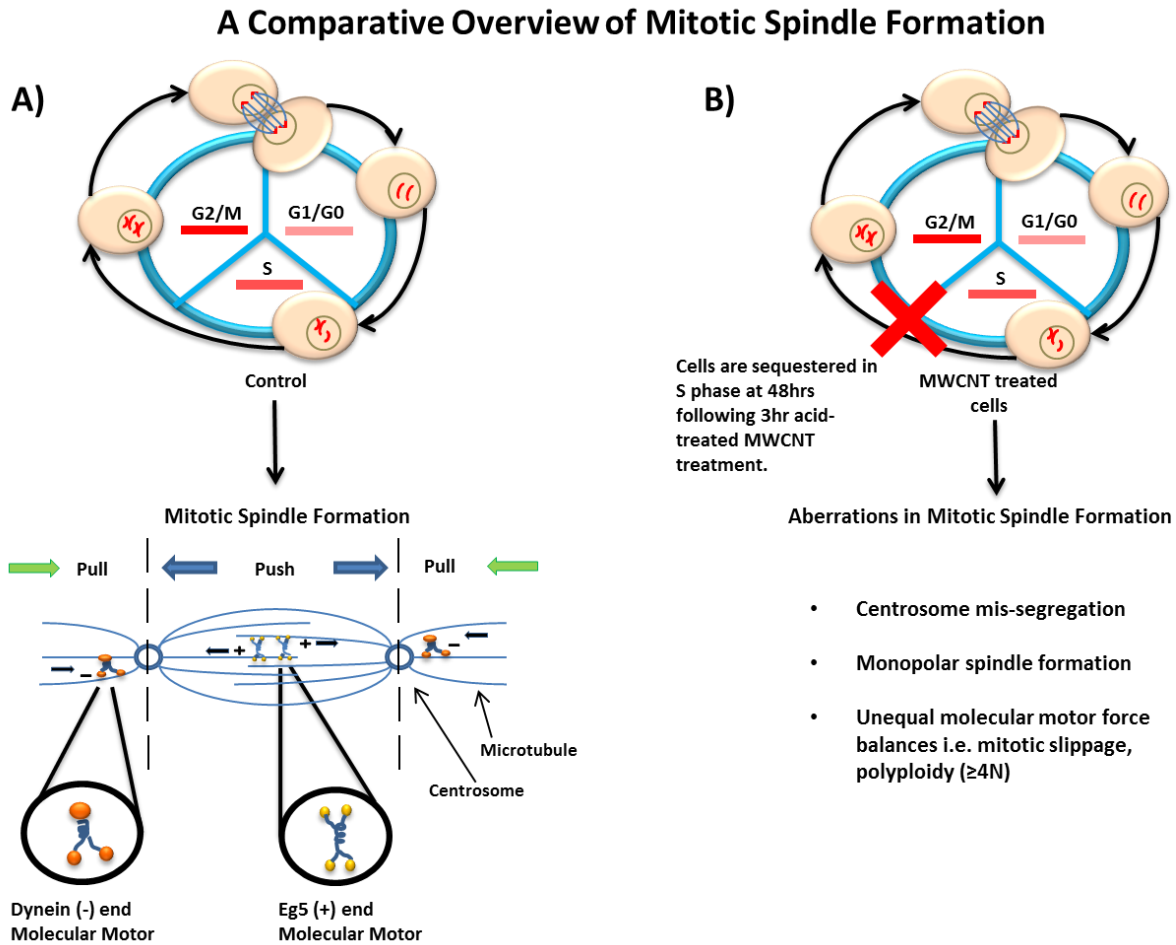


Figure 5: Comparative Overview of Mitotic Spindle Formation-following treatment with MWCNTs the following aberrations are possible with respect to the mitotic spindle assembly relative to the abundance and control of integral mitotic molecular motors, Eg5 and Dynein.

Finally, expression and activity of these integral mitotic molecular motors are regulated through the cell cycle via check-point regulation of cyclin dependent kinases^{53,55,83,122,123}, and of their respective promoter regions^{122,124}. Thus, if critical timings of the cell cycle are affected by exposure to MWCNTs, reductions in mRNA expression of integral molecular motors will follow; our data support this assertion. Further, as studies have reported, MWCNTs have the ability to biopersists¹²⁵, here the concern is that continued exposure could lead to a full

dismantling of the mitotic apparatus, leading to mono and multipolar spindle formation⁹ through fragmentation of the centrosome and breakage of the DNA^{16,49}. Ultimately, this continued cellular insult can result de-regulation^{4,126} of the cell cycle and initiation of aneuploidy¹²⁷.

Conclusion

In conclusion, the present investigations reveal that 24 h MWCNTs exposure (1 and 3 h acids oxidized, at 0.24 µg/ml) induced changes in the BEAS-2B cell cycle. Further, our data is the first to show expressional changes in the integral molecular motor protein dynein and Eg5, key players of the cellular division process. The observed changes are dependent on the MWCNT physical and chemical properties. Disruption of the cell cycle and expression of motor proteins involved in the microtubule spindle formation and cellular division are an early indicator of the potential genotoxicity induced by the carbon nanotube, and MWCNTs-induced cancer progression. This investigation also provides useful information about the possible routes for aneuploidy while highlighting the need for further studies to address the role of cellular motors in the transport of nanomaterials as well as their possible role in tumorigenesis.

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